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(54) Title: METHOD FOR IMMOBILIZING HAPTENS ON A TEST ARTICLE

(57) Abstract

A method for preparing test articles comprises immobilizing a hapten-carrier molecule conjugate on a solid phase surface. Attachment of a hapten is effected through the carrier molecule which non-covalently adsorbs to the surface. The hapten is a small molecule which retains binding activity even under relatively harsh conditions which would degrade more labile substances such as antibodies and other binding proteins. Such labile binding proteins may subsequently be introduced to the solid phase through binding with anti-hapten binding substance under mild conditions. Optionally, the carrier molecule can be covalently attached to the surface after initial non-covalent position, for example by radiation-induced cross-linking.

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METHOD FOR IMMOBILIZING HAPTENS ON A TEST ARTICLE

BACKGROUND OF THE INVENTION

1. Field of the Invention:

The present invention relates generally to the production of test articles used for determining the presence of analyte in biological samples. More particularly, the present invention relates to a method for immobilizing haptens onto solid phase surfaces.

A wide variety of assay techniques and methods exist for determining the amount of a target analyte in a biological or other sample. Specific binding assays rely on detecting an analyte using a specific binding substance which reacts with the analyte in a highly selective manner. Numerous protocols and formats for performing such specific binding assays are described in the patent, technical, and medical literature.

Of particular interest to the present invention, many specific binding assays employ a solid phase surface having a specific binding substance, such an antibody or antibody fragment, immobilized on the surface. The surface may then be utilized to capture analyte through direct or indirect binding of the immobilized specific binding substance to the target analyte.

Immobilization of the specific binding substance on the solid phase surface can be problematic in a number of respects. First, the specific binding substance must be applied and attached to the surface under conditions which do not result in loss of binding activity, e.g. conditions which do not result in denaturing of antibodies or other binding protein. Thus, a variety of otherwise desirable application techniques, such as ink jet printing, are difficult to use since they can denature labile proteins, such as antibodies. Second, many antibody binding protocols which are sufficiently gentle to avoid denaturing the binding substances require

multiple steps which are time consuming and labor intensive, making preparation of the solid phase surface expensive. The expense is further increased when it is desired to form a plurality of discrete, frequently very small, reaction zones on a single solid phase surface. In addition, many specific binding substance immobilization techniques require considerable time to achieve maximum binding of the substance to the solid phase surface. Attempts to shorten the binding time often result in the desorption or sloughing of the substances from the test surface.

For these reasons, it would be desirable to provide improved methods for immobilizing specific binding substances on solid phase surfaces to produce test articles useful for performing biological and other assays. In particular, it would be desirable to provide improved fabrication methods where the specific binding substance can be attached in a rapid manner over very small areas on a surface using accurately and precisely measured volumes of a binding substance to define a reaction zone. It would be further useful to provide methods for immobilizing binding substances which can be performed using conventional printing methods and equipment, such as ink jet printers of a type which can subject the material being deposited to relatively harsh and often denaturing conditions. The methods of the present invention are preferably able to utilize materials which can withstand such denaturing conditions, allowing the preparation of test articles having multiple and precisely defined reaction zone patterns which can be achieved using printing techniques. The ability to employ conventional printing techniques provides for economic fabrication of test articles, even when the test articles require the complex and precise formation of reaction zones on the solid phase test surface.

2. Description of the Background Art

The printing of chemical and biological reagents on surfaces is described in a number of patent publications, including U.S. 4,046,513 (reagents); 4,366,243 (haptens); 4,877,745 (ink jet printing of biological reagents such as

allergens); 5,108,926 (ink jet printing of viable cells); 5,260,195 (ink jet printing of a polymer binder and enzyme coating mass); 5,326,691 (ink jet printing of cells and cell-binding agents); and JP6/041483 (Abstract) (printing inks for jet printers which incorporate proteins to modify appearance). Photolithographic fabrication of biosensor surfaces is described in U.S. Patent Nos. 5,212,050; 5,200,051; and 5,063,081. U.S. Patent No. 5,316,784, describes a two-step process for attaching immunologically active substances, such as antibodies, antigens, and binding proteins, to solid phase surfaces by applying a mixture of the active substance and a linking group having photoactivable groups to the surface. After allowing the mixture to absorb into the surface, the active substance is covalently attached by activating the linking group. U.S. Patent No. 5,258,041, describes the use of spacer arms having hydrophobic guiding groups for attaching biomolecules to solid phase supports.

SUMMARY OF THE INVENTION

Methods for preparing solid phase surfaces for use as test articles in biological and other assays comprise immobilizing a hapten on the solid phase surface via a carrier molecule, typically a macromolecule such as a protein, which is capable of non-specific adherence to the surface. A conjugate, of the hapten and carrier molecule typically covalently attached, is applied to the surface so that the carrier molecule adheres to and the hapten is immobilized precisely at the site of application. The hapten is a small molecule which will retain binding activity even under relatively harsh conditions of application that would denature antibodies and other specific binding proteins. While the carrier molecule, which may be a protein, might be denatured under such conditions, loss of biological activity of the carrier molecule is not significant since it will still provide for the necessary non-specific adherence to the solid phase surface and preservation of the attached hapten.

In some cases, the test articles with immobilized hapten may find use in assays where the hapten is used to bind

directly or indirectly to an analyte of interest, typically where the analyte has previously been bound to anti-hapten antibody. Alternatively, the test article may be further prepared by introducing a specific binding substance, usually an antibody or antibody fragment, to the hapten via an anti-hapten linker moiety. Preferably, a covalently attached conjugate of the specific binding substance and the anti-hapten binding substance may be reacted with the solid phase surface of the test article under relatively mild (non-denaturing) conditions to form a non-covalent linkage between the hapten and the specific binding substance.

Such fabrication methods are preferably performed using printing processes where discrete droplets of a liquid medium containing the hapten-carrier molecule conjugate are applied to the solid phase surface to form precise and accurately defined reaction zones, often in complex geometric patterns which would be difficult to produce using other techniques. The printing processes also allow for application of different haptens within different reaction zones, to produce test articles which are useful in multiple analyte assays. In a preferred aspect of the present invention, particularly ink jet printing is employed where the liquid medium is contained in a reservoir and the droplets are propelled or ejected through an aperture by known ink jet printing technologies. Both piezoelectric and thermal ink jet printing systems may be used for performing the methods of the present invention.

Test articles according to the present invention will have a solid phase test surface having hapten immobilized thereon via a carrier molecule. The carrier molecule will usually be non-specifically attached to the solid phase test surface, but in some cases, it may be desirable to further covalently cross-link the carrier molecule to the surface (using conventional cross-linking methods employed after the initial non-covalent attachment). Such further covalent attachment has several advantages. First, cross-linking of the carrier can reduce the time necessary to achieve high affinity or avidity binding of the complex to the solid phase.

Second, presence of the carrier molecule reduces the risk of denaturing the hapten which provides the active binding site in the complex. Third, the chance of desorption or other loss of the complex is greatly reduced. The solid phase may be composed of any material conventionally used in assay systems, such as organic polymers, glasses, ceramics, metals, absorptive papers, and the like. The haptens and the carrier molecules will generally be as described above.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic illustration of an ink jet printing system adapted for the printing of hapten-carrier molecule conjugates on a solid phase disk surface according to the method of the present invention;

Fig. 2 is an electrical schematic diagram showing the components of the head drive electronics of the ink jet printing system of Fig. 1;

Fig. 3 is an illustration of a biotin-BSA printed disk showing discrete patterns and demonstrating the retention of streptavidin binding activity;

Fig. 4 is a graph illustrating the desorption of biotin-BSA as a function of liquid-phase adsorption time;

Fig. 5 is a graph illustrating the amount of time required for maximal biotin-BSA binding to the acrylic test surface once spotted with the ink jet system;

Fig. 6 is a graph illustrating the acceleration of biotin-BSA binding by heat treatment; and

Fig. 7 is a graph illustrating the results of an assay for CKMB using a solid phase test article prepared according to the present invention, wherein binding of CKMB to the test article is determined both interferometrically and via light absorption.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods are provided for attaching haptens to solid phase surfaces to form test articles useful for detecting target analytes in biological and other assays. The target analyte will be a member of a specific binding pair (SBP),

including compounds, compositions, aggregations, and virtually any other substance which may be detected or reacted by immunological or equivalent techniques. That is, the analyte, or a portion thereof, will usually be antigenic or haptenic, defining at least one epitopic site, or will be a member of a naturally-occurring binding pair (*e.g.*, carbohydrate and lectin, hormone and hormone receptor, ligand and anti-ligand, and the like). Analytes of particular interest include antigens, antibodies, proteins, glycoproteins, carbohydrates, macromolecules, toxins, bacteria, tumor markers, and the like, which define a plurality of epitopic sites. Other analytes of interest include haptens, drugs, and other small molecules, which usually define only a single epitopic binding site. A non-exhaustive list of exemplary analytes is set forth in U.S. Patent No. 4,366,241, at column 19, line 7, through column 6, line 42, the disclosure of which is incorporated herein by reference. Of particular interest to the present invention is the detection of cardiac markers, such as troponin I, troponin T, myoglobin, and creatine kinase isozymes.

The analytes may be present in a wide variety of samples, where the sample is liquid, can be liquified, or can be suspended in a liquid. The methods of the present invention will find their greatest use with biological specimens such as blood, serum, plasma, urine, cerebral fluid, spinal fluid, ocular lens liquid (tears), saliva, sputum, semen, cervical mucus, scrapings, swab samples, and the like, which are frequently employed in the diagnosis and monitoring of disease and therapeutic treatments. In addition, the methods of the present invention may be used with industrial, environmental, and food samples, such as water, process streams, milk, meat, poultry, fish, conditioned media, and the like. In certain circumstances, it may be desirable to pretreat the sample, such as by liquification, separation, solubilization, concentration, filtration, chemical treatment, or a combination of these steps, in order to improve the compatibility of the sample with the remaining steps of the assay, as described hereinafter. The selection and pretreatment of biological, industrial, and environmental

samples prior to immunological testing is well known in the art and need not be further described.

The haptens which are to be immobilized on the solid phase surface are small molecules, typically being below 2000 Daltons, usually being below 1000 Daltons, frequently having a molecular weight in the range from 100 to 500 Daltons, and more frequently having a molecular weight in the range from 200-500 Daltons. Such small molecules will generally be resistant to the denaturing conditions which may be present in the methods of the present invention, particularly when ink jet printing mechanisms are employed, and will thus remain stable and biologically active so that they can bind their specific binding partner. While ink jet printing mechanisms differ significantly in their designs, such mechanisms can subject the material to be deposited to a variety of conditions which can denature labile materials, such as localized heating (including boiling of the liquid medium carrying the material to be deposited), substantial shear forces which can physically disrupt larger molecules, as well as adverse drying conditions utilized after the material has been deposited.

The haptens should possess a high-affinity binding partner, preferably having a binding affinity of at least about 10^{-8} M^{-1}, more preferably being at least 10^{-10} M^{-1}, and most preferably being 10^{-11} M^{-1} or higher. The haptens should be suitable for attachment, usually covalent attachment, to the carrier molecule, as described in more detail hereinbelow. Exemplary haptens include biotin (which binds to avidin, streptavidin and anti-biotin antibody with very high affinity), fluorescein (which binds with high affinity to anti-fluorescein antibody), dinitrophenol (which binds with very high affinity to anti-dinitrophenol antibody), digoxin (which binds with very high affinity to anti-digoxin antibody), luminol (which binds with very high affinity to anti-luminol antibody), theophylline (which binds with very affinity to anti-theophylline antibody), morphine (which binds with very high affinity to anti-morphine antibody), and the like.

Carrier molecules useful in the methods of the present invention will typically be macromolecules having a molecular weight of at least about 10 kiloDaltons (kD) with no upper size limit, usually being at least about 20 kD, and typically being in the range from about 20 kD to 200 kD. Particularly suitable are large proteins which have high non-specific binding affinities for the solid phase surface of interest. Such proteins, however, should not contribute to the non-specific binding of other substances to the surface after they have been immobilized thereon. Exemplary carrier proteins include albumins, ovalbumins, immunoglobulins, thyroglobulins, ferritin, and the like. Particularly preferred carrier proteins include serum albumins, such as bovine serum albumin (BSA), human serum albumin (HSA), and the like.

The hapten and carrier molecule will be conjugated to each other to form a hapten-carrier molecule complex prior to application to the solid phase surface. Such conjugation will usually be achieved through covalent binding using conventional chemistries depending on the natures of both the hapten and the carrier molecule. Frequently, covalent binding will be achieved using a bivalent cross-linking substance which is capable of attaching at one end to the hapten and at the other end to the carrier molecule. Such substances include succinimides, carbodiimides, glutaraldehydes, diazotization coupling, and the like. These and other suitable coupling chemistries are well-described in the biological and chemical literature. See, for example, Avrameus et al. (1978) Scand. J. Immunol. 8:7-23. The resulting conjugates will typically have hapten numbers of at least 1, more usually of at least 2, preferably being at least 4, often being in the range from 4 to 50, and preferably being in the range from 6 to 20.

The hapten-carrier molecule conjugates will be present in a liquid medium suitable for direct application to the solid phase surface of the test article. The liquid medium will typically be aqueous, more typically being buffered within a desired pH range, usually from pH 5 to pH 9.

Phosphate-buffered saline (PBS) is a suitable liquid medium for most hapten-carrier molecule conjugates.

The liquid medium may contain other components for achieving desired purposes. For example, liquid media may include preservatives, such as sugars, intended to enhance stability of the conjugate during application. Surprisingly, however, it has been found that the hapten conjugates of the present invention generally do not require preservative or other components to maintain stability of the haptens in the printing methods of the present invention. The ability to dispense with preservatives (which can interfere with binding of the carrier molecule to the surface) is a particular advantage of the present invention.

The liquid medium will typically have a hapten-carrier molecule concentration in the range from about 1 μg/ml to 250 μg/ml, preferably from 5 μg/ml to 50 μg/ml, with viscosities equivalent to water, preferably being about 1 cp to 2 cp. It is possible to add viscosity-enhancing components, such as glycols, polyvinyl alcohols, or the like, where the viscosity is increased to about 5 to 10 cp, resulting in improved fluid dispersion characteristics from an ink jet printing head. The use of viscosity-enhancing reagents, however, can interfere with protein adsorption particularly to plastics where it will interfere with non-specific hydrophobic interactions between a protein and a plastic surface. Thus in many cases, the addition of such viscosity-enhancing components can decrease the amount of active hapten-carrier molecule adsorbed onto the solid phase surface, and the addition of viscosity enhancing components is not generally preferred at present.

A preselected volume of the liquid medium (typically from 200 pl to 100 μl) containing the hapten-carrier molecule conjugates may be applied to the solid phase surface in any manner that results in an initial non-covalent attachment of the carrier molecule to the surface. For example, the liquid medium may be applied by pipetting, including micropipetting (volumes in the range from about 200 pl to 100 μl), spraying, printing, dipcoating, spin coating, stamping, or the like.

The preferred and exemplary method for applying the liquid media comprises printing using a conventional or modified ink jet printing mechanism where the liquid medium is initially contained in a reservoir and dispensed therefrom in discrete liquid droplets (typically each having a volume in the range from 10 pl to 1 μl) using piezoelectric, thermal, or other dispensing mechanisms.

Ink jet printing technology is well known in the art and described in numerous patents. See, for example, U.S. Patent Nos. 4,240,081; 4,475,113; 4,533,082; 4,544,933; 4,591,883; and 5,164,740, the full disclosures of which are incorporated herein by reference. Typically, the fluid to be printed (which is the liquid medium in the case of the present invention) is ejected through a small aperture under pressure and transformed into uniform droplets by one of various known ink jet printing mechanisms. A preferred class of ink jet printer relies on an evaporative volume increase produced by thermal cycling of the liquid medium. Such ink jet printing mechanisms are available commercially from Hewlett-Packard, Palo Alto, California. Other ink jet printing mechanisms which may find use rely on vibration of a piezoelectric crystal or thermal effect to produce the droplets, where droplets may be charged electrostatically and deflected by a control system, typically a computer. Using either system, the resulting pattern of applied liquid medium can be precisely and accurately controlled. As described in detail in the Experimental section hereinafter, the ink jet printing mechanism will typically be combined with a mechanism for supporting the solid phase surface to be derivatized. In this way, the solid phase can be automatically relocated relative to the ink jet printing head in order to print the liquid medium (which contains the hapten-carrier molecule conjugate) to different locations on the solid phase surface. Thus, a plurality of different reaction zones can be formed on the solid phase surface. Moreover, by employing multiple reservoirs for containing different hapten-carrier conjugates, or alternatively by refilling a single reservoir with different hapten-carrier molecule conjugate(s), reaction zones

having a variety of different activities can be formed on a single solid phase surface.

The solid phase surface employed in the methods of the present invention will include at least a single reaction zone, often including two or more reaction zones, frequently including from 3 to 100 reaction zones, and often including from 4 to 50 reaction zones. The reaction zones may be specific for a single analyte, or different reaction zones may be specific for different analytes. It will be appreciated, of course, that the reaction zones may be capable of directly or indirectly, competitively or non-competitively binding, or providing any other type of binding compatible with the assay formats of the present invention.

The reaction zones are usually optically flat to facilitate spectrophometric, interferometric, fluormetric or other interrogation thereof. The reaction zones will usually have a minimum area of 0.01 mm^2, usually being from 0.1 mm^2 to 100 mm^2, and preferably from 0.1 mm^2 to 5 mm^2. For example, a plurality of circular reaction zones having areas in the range from 0.1 mm^2 to 5 mm^2 may be formed on a single flat surface of a disk substrate. When the reaction zone(s) are formed by the application of discrete droplets of the liquid medium, the total number of droplets will usually be in the range from 1 to 10,000, preferably being from 500 to 2,500, for an approximately 1 mm diameter spot.

The substrates which provide the solid phase surface may be composed of a variety of conventional materials, including organic polymers, glass, ceramics, metals, and the like. For use with interferometers, substrates will preferably be transparent or translucent, and will be composed of organic polymers. An exemplary material is methylmethacrylate polymer.

After applying the liquid medium containing the hapten-carrier molecule conjugate to the solid phase surface, either by ink jet printing or other techniques, the surface may be treated to enhance adherence of the carrier molecule to the surface. It has been found that the solid phase surface may be at a temperature above room temperature, typically at

least 50°C, and often 90°C, or higher, to provide substantially accelerated and enhanced adherence of the carrier molecule to the surface. Such high temperature treatment is typically performed for a relative short period sufficient to maximize binding of the complex to the surface, usually from 10 minutes to 1 hour. While such conditions would inactivate many antibodies and other specific binding materials, the hapten-carrier molecule complexes of the present invention will remain active and able to bind their binding partners even after exposure to heat and other potentially denaturing conditions.

In addition to, or as an alternative to, heating the surface to enhance non-covalent bonding, it is also possible to covalently cross-link the carrier molecules to the solid phase surface by subjecting the adsorbed surface to suitable conditions, *e.g.*, light, radiation, heat, cross-linking reagents, or the like. It should be noted that such covalent cross-linking of the molecule occurs after initial immobilization (usually non-covalent attachment) has been effected. A particular advantage of the present invention is that the hapten may be accurately and precisely adhered to the solid phase surface without the need to previously derivatize the surface for covalent linkage. Under certain circumstances, however, it may be desirable to provide for such covalent linkage after the reaction zones have been initially defined non-covalently.

In a preferred aspect of the present invention, the hapten-carrier molecule will be further derivitized to include a cross-linking moiety, preferably a photo-activatable group such as a benzophenone of fluorinated aryl azide. After adsorption of the complex to the solid phase, usually a plastic surface, ultraviolet radiation can be directed at the solid phase surface to convert the cross-linking groups to reactive intermediates which will insert into the carbon-hydrogen bonds of the plastic surface. In order to accelerate the cross-linking, the complexes will be highly substituted with the reactive groups on the carrier molecule (typically from 1 to 25, preferably from 5 to 15 substitutions per

molecule) and high doses of the ultraviolet radiation can be applied to the surface (typically from 100 to 1,000 $\mu W/cm^2$ at 6 inches from the surface). While denaturation of biologically active materials is likely under these conditions (because of formation of multiple linkages and the random distribution of the covalent bonds to the surface, as well as the susceptibility of many molecules to ultraviolet light), the hapten-carrier molecule complex of the present invention has been found to be significantly less susceptible to such denaturation. The formation of multiple links between the carrier molecule and the plastic surface will not significantly reduce the binding activity of the hapten. Moreover, the hapten and carrier molecule may be selected for optimal ultraviolet light tolerance, preferably both having low absorption in the UV range around 280nm.

After the hapten has been immobilized on the solid phase surface via the carrier molecule, the resulting test article may be used directly in certain assay formats. For example, assays which rely on introducing an anti-hapten binding substance to a target analyte using antibody or other binding substance specific for the target analyte can utilize the test article having the immobilized hapten as a capture device. For example, avidin bound to a target-analyte specific antibody may be reacted with the test sample. The resulting complex of analyte, antibody and avidin may then be captured using a test article having biotin hapten immobilized on its test surface. After separating the solid phase from the test sample, the presence of the target analyte can be confirmed using antibody attached to a label in a conventional "sandwich" assay format.

The test articles of the present invention may alternatively be further derivatized to have a specific binding substance of interest immobilized to the test surface. Such immobilization may be readily accomplished by reacting the solid phase surface under mild (non-denaturing) conditions with a solution containing a specific binding substance, such as an antibody or antibody fragment, covalently attached to the anti-hapten binding substance. In this way, the specific

binding substance is precisely and accurately bound to the reaction zone(s) defined by the immobilized hapten under mild conditions which do not result in loss of activity of the specific binding substances. Such test articles are useful in a variety of conventional assay formats which employ direct binding between the immobilized specific binding substance and a target analyte.

The following examples are offered by way of illustration, not by way of limitation.

Experimental

1. Immobilizing Biotinylated BSA to Acrylic Test Articles

A. Jetting System Description

A system for ink jet printing of haptenated macromolecules is shown in Figs. 1 and 2. The system includes a HP Model 51616A Ink Jet Head 10 (Hewlett Packard Ink Jet Components Division, Corvallis, OR). To facilitate injecting haptenated solutions into the jetting chambers 12 of the head 10 and to avoid contamination of the solutions, the face plate of Model 51616A was obtained sans the molded part which normally forms the ink reservoir. A fluid tube 14 was then easily connected between a 15 mL polypropylene test tube 16 containing the haptenated solution and the entrance to the jetting chamber 12. The head 10 was mechanically and electrically interfaced using a HP Model 51610A Carriage Assembly 18 (Hewlett Packard Ink Jet Components Division, Corvallis, OR). The resulting assembly was mounted on X-axis and Y-axis micrometer slides 20 and 22 to facilitate positioning the head precisely over an acrylic disc D on which the solutions were to be printed. Prior to printing, the acrylic disc D was installed on a round disc mounting platen 24 via insertion on a center locating pin. The disc mounting platen 24 was attached to a motor shaft of stepper motor 26 (Parker-Compumotor, Harrison City, PA, Model # S57-51-MO), allowing the disc to be rotated under computer control. The stepper motor 26 was mounted on a Z-axis slide 30 which allowed a stepper motor 28 (Parker-Compumotor Model # S57-51-MO) to drive the platen 24 in the Z direction. This

facilitated lowering the disc D for removal and allowed the vertical distance between the head and the disc, a critical parameter in reproducible printing, to be replicated run to run. The number of droplets ejected from the head 10 to form each deposited haptenated macromolecule spot, as well as the energy pulses which caused the thermal head to fire were controlled by head drive electronics 31 (see Fig. 2). Stepper motors were controlled using two S-6 Series Microstep stepper motor controllers 32 (Parker-Daedal, Harrison City, PA). The stepper motor controllers 32 were interfaced to computer 34 using an AT-MIO-16X DAQ acquisition board 36 (National Instruments Corporation, Austin, Texas). Control software was written to control the motor motions using a LabVIEW software package (National Instruments Corporation, Austin, Texas).

Fig. 2 shows details of the head drive electronics 31 and of the electrical schematic of the HPO Model 51616A Ink Jet Head 10. The purpose of the head drive electronics is to (1) supply a controlled impulse of energy to a selected thermal resistor 40 inside the ink jet head, (2) control the rate at which pulses are input to the head, and (3) control the number of energy pulses delivered to the head in a sequence thus controlling the amount of fluid deposited in one spot on the acrylic disc D. The circuit functions as follows. The ink jet printing technology relies upon heating the fluid contained in small chambers inside the ink jet head. This heating is accomplished by passing electrical current through small resistors 40 embedded in the fluid chambers. As shown in the schematic, the HP Model 51616A Ink Jet Head 10 has thermal heating resistor values of approximately 65 ohms. Each of the 12 orifices in this head has a heating resistor 40 localized above it. The 12 resistors are connected internal to the head in the network shown in Fig. 2. Internal wiring resistances result in the parasitic resistances also shown in Fig. 2. All chamber heating resistors are connected to one common line. To drive the head, MOSFET transistor 42 (BUZ71; Siliconix, Santa Clara, CA) is turned on under control from NPN transistor 44 (2N3904; Motorola, Phoenix, AZ) when a high logic state is output from monostable multivibrator 46

(74LS123; Texas Instruments, Dallas, Texas). The monostable multivibrator 46 controls the time that MOSFET transistor 42 is allowed to stay on allowing electrical current to pass through the chamber heating resistor 40 in the ink jet head 10.

Resistor 48 (20 kΩ) is adjusted to control the pulse period and tune the head for optimal jetting of the solution of interest. A pulse width between 3 and 3.5 microseconds was found to be optimal for jetting the solutions of interest. The common node of the ink jet head is supplied with a 23 volt DC potential from power supply 50 (Model LPS152, Leader Instruments, Hauppauge, NY). This voltage is held constant under all operating conditions using the monostable multivibrator 46 to control the energy input to the chamber heating resistors 40. The number of pulses of energy supplied to the head 10 to form one spot is controlled by 12 bit pre-loadable binary counter 52 which was loaded with the number of droplets to be printed using 12 SPST switches 54. A 2 KHz clock source 56 is input to the counter 52 and is used to count down to zero from the pre-loaded number. The resulting output occurs at a 1 KHz rate, half the input clock rate. The maximum rate at which the HP 51616A ink jet head 10 has been specified to be driven is 1.25 Khz, though it can be operated up to 3 to 4 Khz for accelerated deposition rates. Even higher jetting rates cause inconsistent jetting and damage to the head (resistor failure). The line connected from the counter 52 to the computer interface 36 is used to command the head drive electronics 31 to deposit a spot on the acrylic disc D after the stepper motors 26 and 28 have positioned the disc D in the appropriate position.

B. Acrylic Disk Preparation

35 mm acrylic disks used for substrate material were obtained from the Germanow-Simon company. They were machined from Hesalite HTC material and were 1.1 or 1.4 mm thick with a high-tolerance 2.4 mm center hole. This material has optical properties that are sufficient for interferometric and/or fluorescent interrogation and protein binding characteristics

enabling immunoassay development on it. Disks were shipped from the vendor laminated on both sides to minimize scratching during machining, transport, and storage. Prior to ink jet spotting, laminates were removed, and the disks were (occasionally washed with mild detergents and) rinsed with deionized water both to quench static electricity build-up and to remove debris remaining from the machining process.

C. Preparation of Biotinylated BSA solutions

Bovine serum albumin (BSA) was covalently attached to biotin moieties (biotinylated) starting with 60 mg of a 10 mg/mL solution of BSA in phosphate buffered saline (PBS, 10 mM, pH 7.4,). The BSA was then conjugated at a molar ratio of ca. 10:1 NHS-LC-biotin to BSA. For 60 mg of BSA, 4.1 mg of NHS-LC-Biotin (Pierce #94052374) was dissolved in 60 μL of DMF (Pierce #931026155) and added to the 6 mL of BSA in PBS. This reaction mixture was placed in an ice bath for 2 hours followed by placing the mixture in a 12,000 MWCO dialysis tubing and dialyzing against 0.1 M phosphate buffer (pH 7.0, with at least two 1 L changes) at 4°C to remove unreacted biotin. Following dialysis and hapten number assessment, 0.1% NaN_3 was added to the concentrated B-BSA to discourage microbial growth. Hapten numbers in the range from 2 to 9 were obtained. Before spotting these solutions using the ink jet system, an aliquot of a concentrated biotin-BSA solution was diluted to 40 μg/mL in PBS and degassed.

In results to be discussed below, the biotin-BSA has been further derivatized to include a benzophenone moiety that may be activated with ultraviolet light to form a covalent bond with the acrylic test surface. To prepare this Biotin-BSA-benzophenone, 1.0 mL of biotin-BSA (5 mg/mL in PBS) was combined with 68 μL of LC-SPDP (Pierce #21652, 10 mg/mL in DMF) and allowed to react for two hours at room temperature, followed by overnight dialysis vs. PBS. The following day, 40 μL of 100 mM dithiothretol was added to the B-BSA-SPDP and the reaction proceeded for 1 hour at room temperature before being purified on a PD 10 column (Pharmacia) in PBS. The B-BSA-SH was collected in a 2.0 mL fraction. Titration for SH content

with Ellman's reagent showed an SH/BSA ratio of 8.0. To the B-BSA-SH fraction (4.75 mg / 1.95 mL PBS), 50 μL (8.8 mg/mL in DMF) of benzophenone-4-maleimide (Molecular Probes #B-1508, Lot 8A-5) was added. This mixture reacted for two hours at room temperature. Again titrating for SH content, it was found that the residual SH/BSA content was 0.36, indicating that benzophenone incorporation was near 7.5 per BSA. The balance of the Biotin-BSA-Benzophenone material purified by overnight dialysis vs. PBS.

D. Application of Biotinylated BSA solutions to Acrylic Test Articles

While the method of detection places constraints on the spot size and spotting format chosen, Fig. 3 (where protein adsorption is visualized via SA-HRP binding to adsorbed biotin-BSA, followed by metal DAB precipitation - representative materials and methods below) shows that liquid volumes in the range of 200 pL to 200 nL, with corresponding spot sizes in the 0.05 (1 droplet) to 1.0 mm (1000 droplets) range, may easily be achieved in a desired pattern using this embodiment of ink jet technology. Moreover, the use of multiple printing heads allows the formation of complex patterns of different materials which can be deposited simultaneously onto the test surface. For the interferometric detection scheme preferred in this invention, acrylic test disks were typically spotted with 40 μg/mL B-BSA in PBS in 32 or 64 circumferentially-spaced spots per revolution (at 0.4 inches radius) with a drop-per-spot setting ensuring 50% coverage of surface along the circumference.

E. Washing Protein-coated Articles to Remove Excess Materials.

Washing of excess buffer/protein materials (typically with PBS/0.1% Tween 20) from ink jet spotted disks is required so that only an adsorbed (or covalently-attached) monolayer (or a few monolayers) of B-BSA is left on the acrylic surface for subsequent chemistry steps. This washing process removes most of the protein that was originally spotted (ca 99%). To minimize spot smearing, one needs to promote desorption off of the disk rather than across the disk. Modifying the washing solution, and hence the solvency of it, can be coupled with vigorous fluid - disk contact to optimize this cleaning process. Allowing disks to dry before washing also lessens smearing problems. Once spotted, dried, and washed, the protein-coated disks were stable for (at least) several weeks for use in immunoassay development.

F. Retention of Biotinylated BSA to Acrylic Test Articles

1. Liquid-phase adsorption as an example

Classically, protein adsorption is performed under stagnant liquid-phase conditions, where binding between the protein and the surface is achieved over a several hour time scale (at room temperature) and the tightness of binding, or resistance to desorption, increases with adsorption time (Figure 4).

Materials:

Biotinylated-BSA: see "**C. Preparation of Biotinylated BSA solutions**" above. This material was diluted to 40 μg/mL in PBS before use.

Assay Diluent: 0.1% BSA (Pentax), 0.05% Tween® 20 (Mallinckrodt) in 10 mM PBS, 0.2 μm filtered (Gelman acrodisc).

Wash Buffer: 0.1% Tween® 20 (Mallinckrodt) in 10 mM PBS.

SA-AP Conjugate: Streptavidin-alkaline phosphatase (SA-AP, Pierce, Product # 21324) was reconstituted to 1 mg/mL and frozen. Prior to use, the stock was further diluted in the 1:1000 to 1:1000000 range with Assay Diluent.

PNPP Kit: a tablet of PNPP (Sigma, N-2770) was reconstituted in the suggested buffer immediately prior to use.

Rings: Rings several millimeters thick and 3/8 in (I.D.) diameter were affixed to the clean, unspotted, 35 mm acrylic test articles (Germanow-Simon) with double-stick tape to facilitate containment of liquid materials above the test surface.

Procedure:

1) Clean disks with affixed rings had 500 μL of biotinylated BSA added to each well.

2) At various time afterward, the excess biotinylated BSA was removed and the wells were rinsed several times with Wash Buffer.

3) Wells were then filled with 500 μL of Assay Buffer for various times to promote desorption of previously adsorbed materials. Once the desorption phase was complete, the wells were rinsed with deionized water and allowed to dry.

4) The SA-AP conjugate (at a 1:200 dilution to 5 μg/mL) was then added to the wells on the disks in a submilliliter volume (typically 250 μL) and gentle mixing was initiated.

5) After a predetermined time (typically the 60 to 90 minutes required for the reaction to come to completion), excess SA-AP was removed from the wells and

the wells were then washed with a back-and-forth motion in two cups of wash buffer (several strokes in each cup).

6) The metal PNPP substrate (diluted as instructed) was then added to the wells (150 μL) and allowed to react for 20 minutes without agitation.

7) The reaction was stopped by adding 3 N NaOH (50 μL per well).

8) A Molecular Devices microplate reader (E_{MAX}) was used to determine absorbance at 405 nm.

Results:

The results are set forth in Figure 4.

2. Adsorption after ink jetting, normal drying

The protein-binding process after ink jet-based deposition of protein-based solutions onto the acrylic disk actually consists of a least two phenomena - visual drying of the spotting fluid and the associated process of adsorption (or covalent linking, with associated UV-curing chemistry) of a portion of the spotted B-BSA to the acrylic surface. Disk drying time depends on spot size, fluid composition, temperature, and humidity. For water-based vehicles spotted at ambient conditions in the 32 spot format (ca. 0.2 μL per spot), the crusting time is on the order of 10 to 30 minutes. Adsorption, as indirectly measured by SA-HRP-DAB assay after ink jet deposition (Figure 5) occurs on a longer (several hour) time scale that is not unlike that for liquid phase adsorption (Figure 4).

Materials:

Assay Diluent: 0.1% BSA (Pentax), 0.05% Tween® 20 (Mallinckrodt) in 10 mM PBS, 0.2 μm filtered (Gelman acrodisc).

Wash buffer: 0.1% Tween® 20 (Mallinckrodt) in 10 mM PBS.

SA-HRP Conjugate: Streptavidin-horse radish peroxidase (SA-HRP, Pierce, Product No. 21127) was reconstituted to 1 mg/mL and frozen. Prior to use, the stock was further diluted in the 1:1000 to 1:1000000 range with Assay Diluent.

Metal Enhanced Diaminobenzidine (DAB) Kit: (Pierce, #34065) a 10X metallic solution was diluted in the provided hydrogen peroxide diluent about 1 hour prior to use.

Rings: One with a diameter similar to the outer diameter of the disk and about 2 mm thick, and the other with an approximately 0.5 inch diameter (and similar thickness) were affixed with double stick tape. This allowed containment of liquid between the rings in order to perform the assay.

Procedure:

1) Spotted, washed (after various amounts of post-spotting time), and dried disks had two rings affixed to them with double-stick tape and then were placed on a rotary mixer.

2) The SA-HRP conjugate (at a suitable dilution) was then added to the disks in a submilliliter volume (typically 0.5 mL) and gentle mixing was initiated.

3) After a predetermined time (typically the 60 to 90 minutes required for the reaction to come to completion), excess SA-HRP was removed from the disk and the disks were then gently washed with a back-and-forth motion in two cups of wash buffer (several strokes in each cup).

4) The metal DAB precipitating enzyme substrate was then added to the disk and allowed to react for a set time in the range of 10 to 30 minutes without agitation.

5) The reaction was stopped by DI-water washing the disks to remove the metal DAB solution. After washing, some disks were dried with heat (typically, a hair dryer).

6) An interferometer was used to determine the amount of material precipitated and attached to the disk, as described in detail in copending application serial number 08/298,998, the disclosure of which is incorporated herein by reference.

Results:

The results are set forth in Figure 5.

3. Adsorption after ink jetting, heat acceleration

Heating (in the 70º to 90º C regime, with the upper temperature limited by the softening of the acrylic surface) after the spotting process accelerates the protein binding process from hours to minutes. For example, heating freshly spotted disks to 90º C for 30 minutes and then developing them with the SA-HRP-DAB assay shows that essentially the same interferometric signal is attained as for disks that had been aged at room temperature for several days. In addition, heat treating aged disks in a similar fashion has little, if any, influence on the eventual interferometric signal (Figure 6). In other words, hours of ambient drying/adsorption are apparently functionally equivalent to minutes of heating. This suggests that either a similar phenomena is occurring due to the heating process (time - temperature superposition) or different mechanisms (such as crosslinking, etc. for the heated samples) are occurring but the surface area for protein - plastic interactions governs maximal binding.

Materials and Procedure:

Same as "**2. Adsorption after ink jetting, normal drying,**" above, except that the disks were heated in a small oven in the 70º to 90º C range for 10 to 30 minutes after spotting and before the disk washing process.

Results:

The results are set forth in Figure 6.

4. Protein binding after adsorption, UV-induced crosslinking

To accelerated the binding process and to maximize retention of proteins to the acrylic test surface, it is prudent to covalently link at least a portion of the deposited proteins to the test surface using bifunctional linking molecules (see "**C. Preparation of Biotinylated BSA solutions**" above for details on preparing benzopohenone derivatized biotinylated-BSA). Table 1, below, presents data illustrating that about 10 minutes of UV illumination is sufficient to bind benzophenone-derivatized B-BSA to a level equivalent to overnight adsorption of B-BSA and that this covalently bound material is highly resistant to detergent-induced desorption.

Materials:

Biotinylated-BSA: see "**C. Preparation of Biotinylated BSA solutions**" above. This material was diluted to 40 μg/mL in PBS before use.

Bennzophenone-derivatized, Biotinylated-BSA: see "**C. Preparation of Biotinylated BSA solutions**" above. This material was diluted to 40 μg/mL in PBS before use.

Assay Diluent: 0.1% BSA (Pentax), 0.05% Tween® 20 (Mallinckrodt) in 10 mM PBS, 0.2 μm filtered (Gelman acrodisc).

Wash Buffer: 0.1% Tween® 20 (Mallinckrodt) in 10 mM PBS.

SA-HRP Conjugate: Streptavidin-horse radish peroxidase (SA-HRP, Pierce, Product #21127) was reconstituted to 1 mg/mL and frozen. Prior to use, the stock was further diluted in the 1:1000 to 1:1000000 range with Assay Diluent.

o-Phenylenediamine Dihydrochloride (OPD) substrate: A solution of 0.4 mg/mL OPD was made by dissolving a 15 mg tablet of OPD (Sigma) into 37 mL of 0.1M citrate-phosphate buffer, pH 5.0. Immediately prior to use, 10 mM hydrogen peroxide was added (37 μL of 30% solution, Sigma) as an activating agent. Sulfuric acid (2N) was added in an in an equal volume to OPD to stop the reaction at the desired time.

Rings: Teflon Rings several millimeters thick and 0.25 inches in inner diameter were affixed to the clean, unspotted, 35 mm acrylic test articles (Germanow-Simon) with double-stick tape to facilitate containment of liquid materials above the test surface. After the protein adsorption step in the assay, the rings were removed and a fresh ring and double-stick tape was affixed to the original double-stick tape. This eliminates assay activity due to "wall effects."

Procedure:

1) Several clean disks with 2 affixed rings each had 100 μL of biotinylated BSA added to one well and 100 μL of benzophenone-derivatized biotinylated BSA added to the other.

2) Half of the spotted disks were UV-illuminated (312 nm maximum, 5 Stratagene Stratalinker bulbs, product #400187) for 10 minutes.

3) 2/3 of the disk lot was then washed several times with Wash Buffer to remove excess biotinylated BSA and then the wells were rinsed several times with deionized water. Half of these disks were then stored while the other half were contacted 12 hours with Wash Buffer in a paddle washer (46 rpm) to stimulate desorption.

4) The remaining 1/3 of the disk lot was stored overnight with the biotinylated BSA solutions still in

contact with the disk surface. They were then washed several times with Wash Buffer to remove excess biotinylated BSA and then the wells were rinsed several times with deionized water.

5) Teflon rings were then replaced with new ones and a new piece of double-stick tape.

6) The SA-HRP conjugate (at a 1:50000 dilution) was then added to the wells on the all the disks in a 100 μL volume.

7) After 60 minutes, excess SA-HRP was removed from the wells and the wells were then washed with a back-and-forth motion in two cups of wash buffer (several strokes in each cup).

8) The freshly-prepared OPD substrate was then added to the wells (50 μL) and allowed to react for 10 minutes without agitation.

9) The reaction was stopped by adding 50 μL of 2 N H_2SO_4.

10) A microplate reader was used to determine absorbance at 490 nm.

Results:

The results are set forth in Table 1.

TABLE 1

Acceleration of biotin-BSA binding, and resistance to detergent-induced desorption, by UV photoactivation. SA-HRP-OPD Assay - see text.

	A-490 Signal	
	Normal	UV Treated
5 minutes protein contact:		
B-BSA	0.434	0.617
B-BSA-Bph	0.619	0.720
5 minutes protein contact, then overnight desorption:		
B-BSA	0.245	0.349
B-BSA-Bph	0.281	0.761
Overnight (ca. 15 hours) protein contact:		
B-BSA	0.736	0.719
B-BSA-Bph	0.609	0.675

5. CKMB Assay Performed using Invention

A preferred embodiment of this invention is to determine CKMB levels in suspected AMI patients. This method, assayed spectrophotometrically or interferometrically, is able to rapidly detect CKMB in clinically-significant levels (Figure 7).

Materials:

Assay Diluent: 0.1% Bovine Serum Albumin (BSA) (Pentax), 0.05% Tween® 20 (Mallinckrodt) in 10 mM PBS, pH 7.4, 0.2 μm filtered (Gelman acrodisc).

Blocking Buffer: 0.3% BSA (Pentax), 0.05% Tween® 20 (Mallinckrodt) in 10 mM PBS, pH 7.4; 0.2 μm filtered (gelman acrodisc).

Wash Buffer: 0.05% Tween® 20 (Mallinckrodt) in 10 mM PBS, pH 7.4.

Ink-jet Spotted Disk with Rings: 32 spot disks that have been printed, washed and had rings affixed for fluid containment. See "**2. Adsorption after ink jetting, normal drying.**"

Streptavidin-antiCKMB Conjugate: Streptavidin/antiCKMB conjugate was prepared using S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA, Pierce #26102), a heterobifunctional linking reagent from Sigma and Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Pierce #22320) from Pierce. Fifteen (15) molar excess of SATA (dissolved at 5 mg/mL in dimethylformamide (DMF) from Pierce) was reacted with 1 mg of antiCKMB at 0.74 mg/mL in PBS at pH 7.4 for 3 hours at room temperature. Also, 15 molar excess of SMCC (dissolved at 5 mg/mL in DMF) was reacted with 1.1 mg of streptavidin at 1.1 mg/mL in PBS for 3 hours at room temperature. Unreacted linkers were removed from both antiCKMB/SATA and streptavidin/SMCC reaction mixtures using Sephadex® G25M column (PD10 column from (Pharmacia) and eluting with 3 mL of 10 mM PBS at pH 7.4. The purified antiCKMB-SATA and streptavidin-SMCC were mixed at the protein molar ratio of 1:3, resulting in the total reaction volume of 6mL. The conjugation reactin was initiated by adding 1 M hydroxylamine to a final concentration of 100 mM and incubated over 18 hours at 4°C. The reaction was stopped by adding 100 mM N-ethylmaleimide (NEM) from Aldrich Chemical at 1 mM final concentration in the reaction mixture and incubating for 15 minutes at room temperature. After the incubation with NEM, the conjugation reaction mixture was concentrated 3-fold, and the final volume was reduced to 2 mL using a Centricon-100 concentrator from Amicon. Then the concentrated mixture was purified using a Bio-Gel® A-5m column from Bio-Rad to isolate streptavidin-antiCKMB conjugate.

Anti-CKMM-Horse Radish Peroxidase (HRP) Conjugate: AntiCKMM-HRP conjugate was made in two steps as follows. First, goat antiCKMM antibody (BiosPacific #G31520) was conjugated to SATA (Sigma) at a molar ratio of SATA to antibody of 20:1. 4 mg of antibody required 0.1156 mg of SATA, which was obtained by adding 5.2 mg of SATA to

500 μL of DMF (Pierce) for a 10.4 mg/mL solution. The 4 mg of goat antiCKMM was diluted to 2.5 mM with PBS, pH 7.3, and 11 μL of SATA was added and incubated at room temperature for 3 hours. HRP (Sigma) was conjugated to SMCC (Pierce) at a molar ratio of SMCC to HRP of 20:1. 10 mg of HRP was diluted to 2.5 mL in PBS, pH 7.3 10 mg of HRP required 1.67 mg of SMCC. 65 mg of SMCC was dissolved in 100 μL of DMF for a 65 mg/mL solution. 26 μL of the SMCC solution was added to the HRP in PBS and incubated for 3 hours.

Before the second step, the HRP/SMCC and goat antiCKMM/SATA were each placed on a PD-10 column (Pharmacia) which had been equilibrated in PBS, pH 7.3 to remove the free SATA and SMCC. The goat antiCKMM was eluted with 3 mL of PBS while the HRP was eluted with 2.8 mL of PBS. This 5.8 ml volume was placed together and 580 μL of 1M hydroxylamine, pH 7.0 (Sigma) was added to start the conjugation which continued at 4°C overnight. The reaction was then stopped by adding 64 μL of a 12.5 mg/mL solution of NEM (Aldrich) in DMF and incubating for 15 min. at room temperature. The 6+ mL were then concentrated in a Centricon 30 concentrator to 2 mL and fractionated over a 100 cm Bio-Gel® A-5M column.

<u>CKMB Calibrators</u>: CKMB (Fortron, #I-028) was diluted to prepare 0, 10, 100 ng/mL calibration solutions.

<u>HRP Substrates</u>:

A) *o-Phenylenediamine Dihydrochloride* (OPD), 0.4 mg/mL made by dissolving 15 mg tablet of OPD (Sigma) into 37 mL of 0.1M citrate-phosphate buffer, pH 5.0. Just prior to addition onto the disk, 10 mM hydrogen peroxide was added (37 μL of 30% solution, Sigma). Sulfuric acid (2N) was used to stop the reaction at the desired time.

B) *Metal Enhanced Diaminobenzidine* (DAB) *kit* (Pierce, #34065). A concentrated 10X metallic solution was diluted in hydrogen peroxide diluent (0.15 mL of concentrate was added to 1.35 mL of diluent).

Procedure:

1) 200 μL of blocking buffer was added to the annulus of each disk. All disks were then placed in 37°C incubator for 10 minutes and mixed with rotomixer.

2) All disks were then washed in Wash Buffer with a back-and-forth motion in several cups.

3) 200 μL of diluted streptavidin-antiCKMB was added into each disk. All disks were placed back onto the rotomixer in the incubator for another 10 minutes.

4) All disks were again washed in Wash Buffer.

5) 200 μL of the CKMB calibrators were added into the disks. All disks were again incubated for 10 minutes as described above.

6) All disks were again washed in wash buffer.

7) 200 μL of diluted antiCKMM-HRP was measured into each disk. All disks were again incubated for 10 minutes as in previous manner.

8) All disks were again washed in wash buffer.

9) Enzyme substrate was added at room temperature.

A) 100 μL of the hydrogen peroxide activated OPD was added onto each of the disks in the first set.

B) 200 μl of the diluted Metal DABN solution was added onto each of the discs in a second set.

10) Following a 10 minutes incubation, the OPD disks were stopped with the addition of 100 μl of the 2N sulfuric acid and the DAB disks were washed off/immersed in three cups of distilled water.

11) The OPD coloring solution (200 μL) was transferred to a microplate and read with microplate reader (Molecular Devices, E_{MAX}) at 490 nm. The DAB disks were dried and measured with the interferometer.

Results:

The results are set forth in Figure 7.

Although the foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for immobilizing a hapten on a solid phase surface, said method comprising applying to the surface a liquid medium including the hapten attached to a carrier molecule under conditions so that the carrier molecule adheres to the surface and the hapten remains active and sterically available for binding to a specific binding partner.

2. A method as in claim 1, wherein the hapten is a small molecule having a molecular weight below 2000 Daltons and the carrier molecule is a macromolecule having a molecular weight above 10 kD.

3. A method as in claim 2, wherein the hapten is selected from the group consisting of biotin, fluorescein, dinitrophenol, digoxin, luminol, theophylline, and morphine.

4. A method as in claim 2, wherein the macromolecule is a protein selected from the group consisting of albumins, immunoglobulins, thyroglobulins, and ferritin.

5. A method as in claim 1, wherein the hapten is covalently attached to the carrier molecule.

6. A method as in claim 5, wherein the hapten is covalently attached through at least one linking moiety.

7. A method as in claim 1, further comprising cross-linking the carrier molecule to the solid phase surface after the carrier molecule has initially adhered by non-covalent interaction.

8. A method as in claim 1, wherein the liquid medium is applied as a spray of discrete droplets each having a volume in the range from 10 pl to 1 μl.

9. A method as in claim 1, wherein the liquid medium is applied by pipetting a volume of the liquid medium in the range from 200 pl to 100 μl.

10. A method as in claim 1, wherein the hapten is immobilized over a discrete reaction zone on the solid phase surface, having an area in the range from 0.1 mm^2 to 100 mm^2.

11. A method as in claim 1, wherein a plurality of discrete reaction zones are formed in a preselected pattern on the solid phase surface.

12. A method as in claim 1, wherein the solid phase surface is composed of a material selected from the group consisting of organic polymers, glasses, ceramics, metals, and paper materials.

13. A method as in claim 1, further comprising binding a specific binding substance to the hapten on the solid phase.

14. A method as in claim 13, wherein the specific binding substance is attached to a hapten-binding substance and wherein binding occurs through the hapten and the hapten-binding substance.

15. A method for immobilizing a hapten on a solid phase surface, said method comprising:

providing a hapten attached to a carrier molecule present in a liquid medium; and

applying a preselected volume of the liquid medium against the solid phase surface so that the carrier molecule non-covalently adheres to said surface to form a reaction zone; and

cross-linking the adhered carrier molecule to the solid phase surface.

16. A method as in claim 15, wherein said cross-linking step comprises exposing the solid phase surface to radiation which effects cross-linking between a moiety attached to the carrier molecule and the surface.

17. A method as in claim 15, wherein the volume of liquid medium is applied by spraying a plurality of droplets having individual volumes in the range from 10 pl to 1 μl propelled by maintaining the liquid medium in a chamber and inducing pressure pulses to force the droplets through an aperture toward the solid phase.

18. A method as in claim 15, wherein the volume of liquid medium is applied by pipetting a volume in the range from 200 pl to 100 μl.

19. A method as in claim 15, further comprising heating the solid phase surface to a temperature and for a time selected to enhance adherence of the carrier molecule to the surface.

20. A method as in claim 15, wherein the aperture is moved relative to the solid phase surface in order to immobilize hapten over a discrete reaction zone on the solid phase surface, having an area in the range from 0.1 mm^2 to 5 mm^2.

21. A method as in claim 20, wherein from 1 droplet to 10,000 droplets are propelled against the solid phase surface to produce each reaction zone.

22. A method as in claim 17, wherein the aperture is moved relative to the solid phase surface in order to form a plurality of discrete reaction zones in a preselected pattern on the solid phase surface.

23. A method as in claim 15, wherein the hapten is a small molecule having a molecule weight below 2000 Daltons and the carrier molecule is a macromolecule having a molecular weight above 10 kD.

24. A method as in claim 23, wherein the hapten is selected from the group consisting of biotin, fluorescein, dinitrophenol, digoxin, luminol, theophylline, and morphine.

25. A method as in claim 23, wherein the macromolecule is a protein selected from the group consisting of serum albumins, immunoglobulins, thyroglobulins, and ferritin.

26. A method as in claim 15, wherein the hapten is covalently attached to the carrier molecule.

27. A method as in claim 16, wherein the moiety is selected from the group consisting of benzophenome and fluorinated aryl azide, and wherein the exposing step comprises exposing the surface to ultraviolet radiation.

28. A method as in claim 15, wherein the solid phase surface is composed of a material selected from the group consisting of organic polymers, glasses, ceramics, metals, and paper materials.

29. A method as in claim 15, further comprising binding a specific binding substance to the hapten on the solid phase.

30. A method as in claim 28, wherein the specific binding substance is attached to a hapten-binding substance and wherein binding occurs through the hapten and the hapten-binding substance.

31. A test article comprising:

a solid phase having a test surface;

hapten attached to a carrier molecule immobilized to the test surface wherein the hapten is linked to the surface by the carrier molecule.

32. A test article as in claim 31, wherein the solid phase surface is composed of a material selected from the group consisting of organic polymers, glasses, ceramics, metals, and paper materials.

33. A test article as in claim 31, wherein the hapten is a small molecule having a molecular weight below 2000 Daltons and the carrier molecule is a macromolecule having a molecular weight above 10 kD.

34. A test article as in claim 33, wherein the hapten is selected from the group consisting of biotin, fluorescein, dinitrophenol, digoxin, luminol, theophylline, and morphine.

35. A test article as in claim 33, wherein the macromolecule is a protein selected from the group consisting of serum albumins, immunoglobulins, thyroglobulins, and ferritin.

36. A test article as in claim 31, wherein the hapten is covalently attached to the carrier molecule.

37. A test article as in claim 36, wherein the hapten is biotin covalently attached to a serum albumin carrier molecule.

38. A test article as in claim 31, wherein the carrier molecule is non-covalently attached to the test surface.

39. A test article as in claim 31, wherein the carrier molecule is covalently cross-linked to the solid phase surface.

40. A test article as in claim 31, further comprising a specific binding substance attached to the hapten by a hapten-binding substance, wherein the specific binding substance and hapten-binding substance are covalently attached to each other.

41. A test article as in claim 31, wherein the hapten is biotin, the carrier molecule is a serum albumin, the hapten-binding substance is avidin, and the specific binding substance is an antibody or antibody fragment.

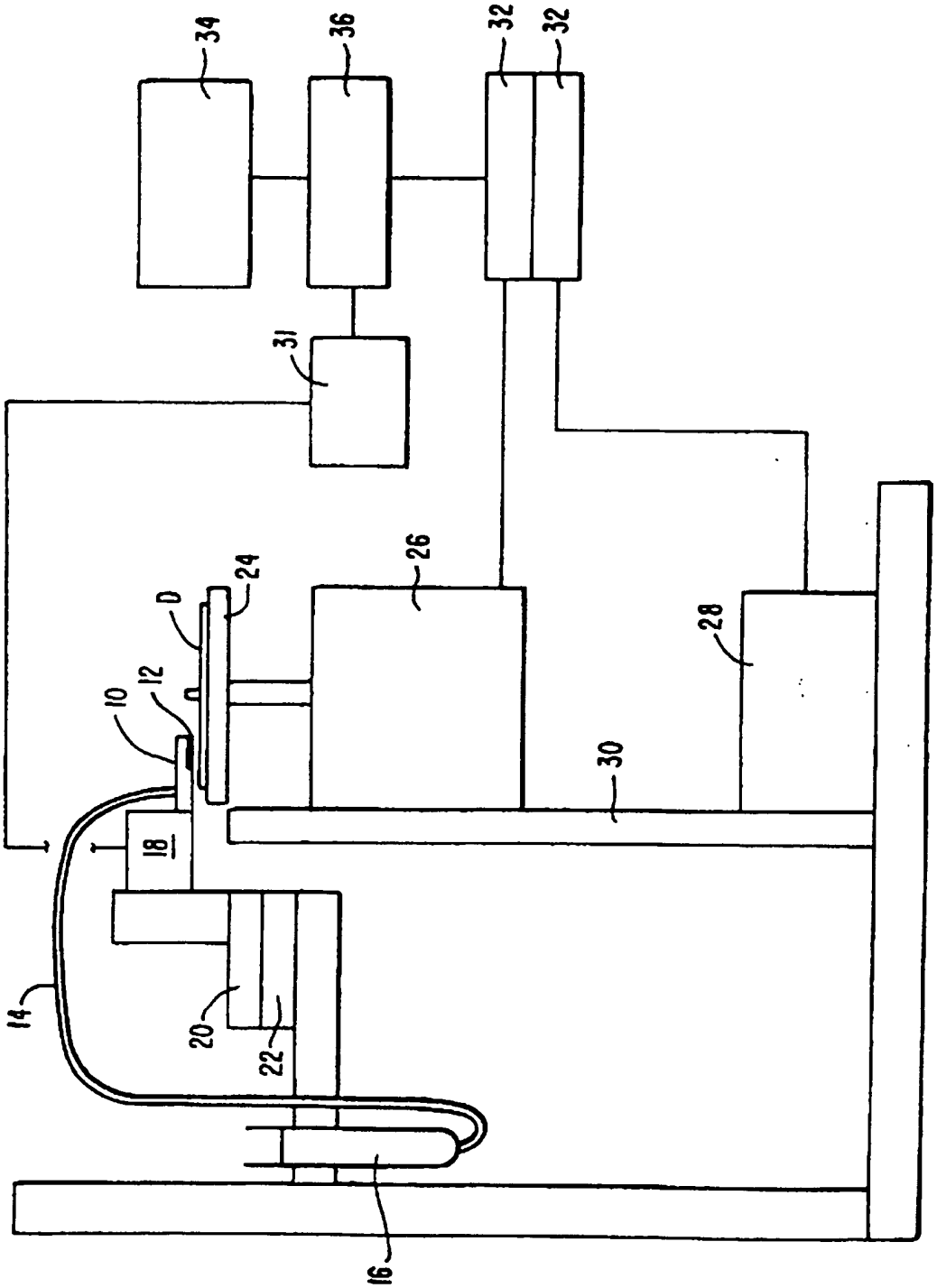

FIG. 1.

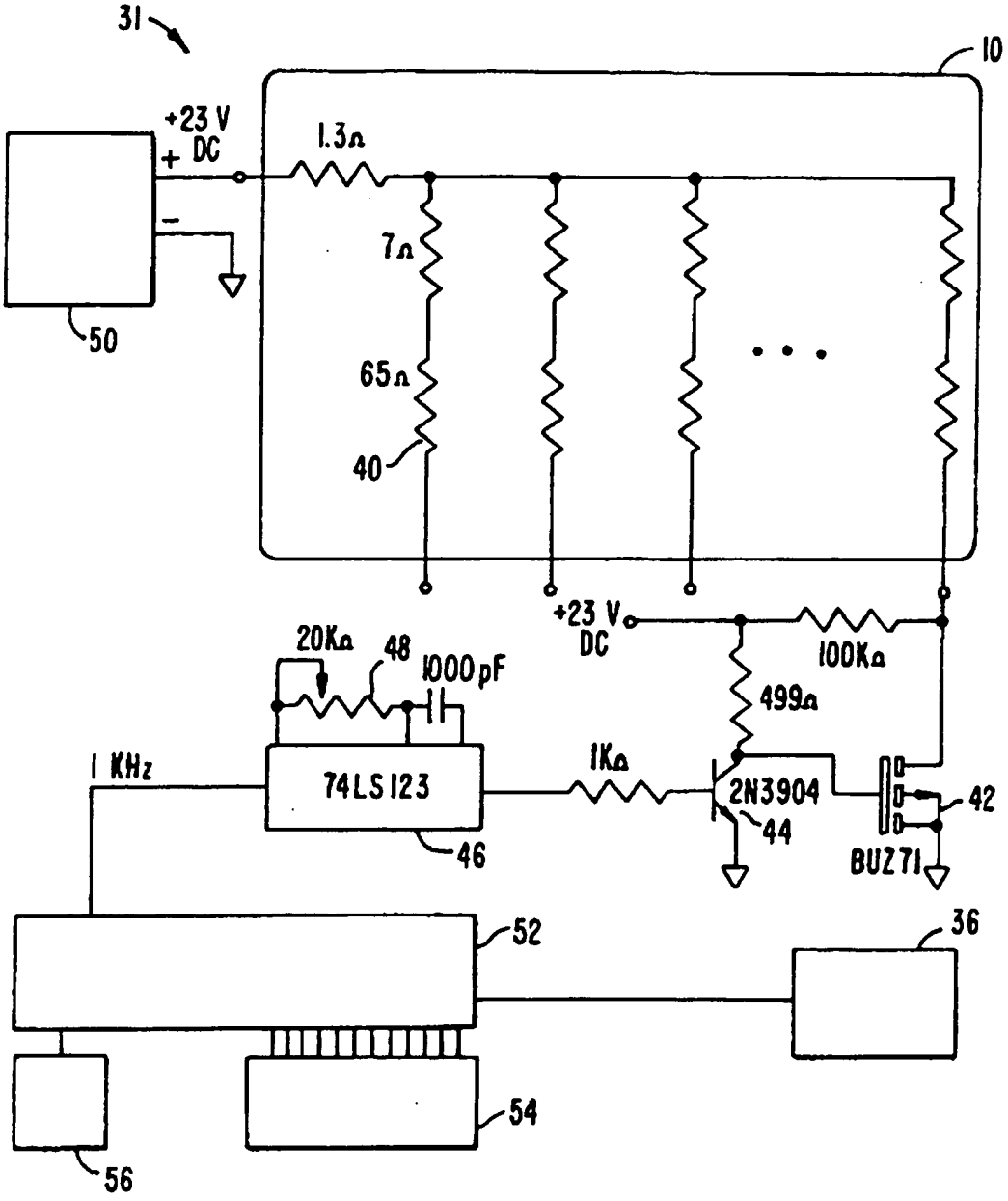
31
10
+23 V
+ DC
1.3Ω
7Ω
65Ω
40
50
+23 V
DC
100KΩ
499Ω
20KΩ
48
1000 pF
1 KHz
74LS123
1KΩ
2N3904
44
42
BUZ71
46
52
36
54
56

FIG. 2.

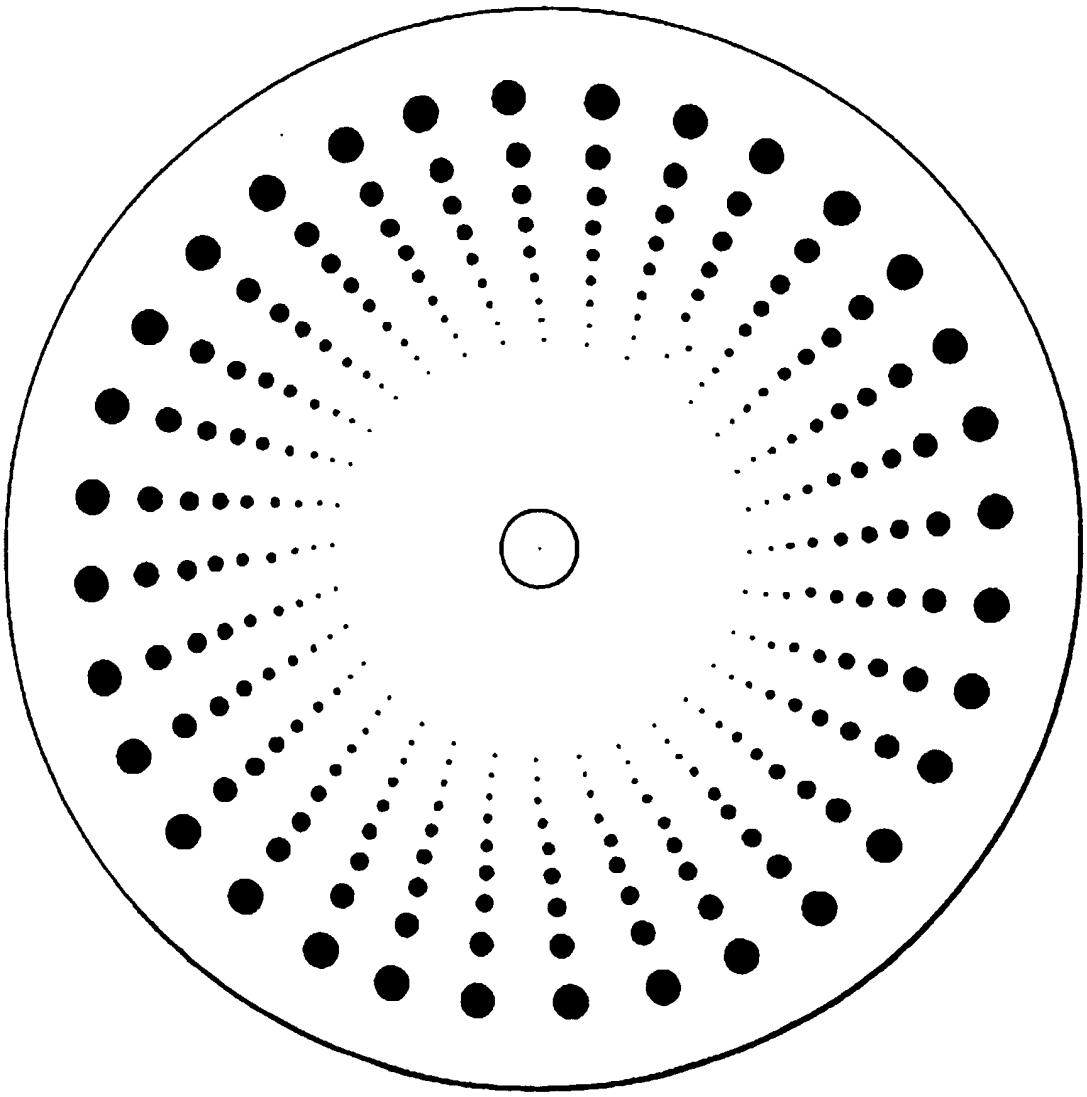

FIG. 3.

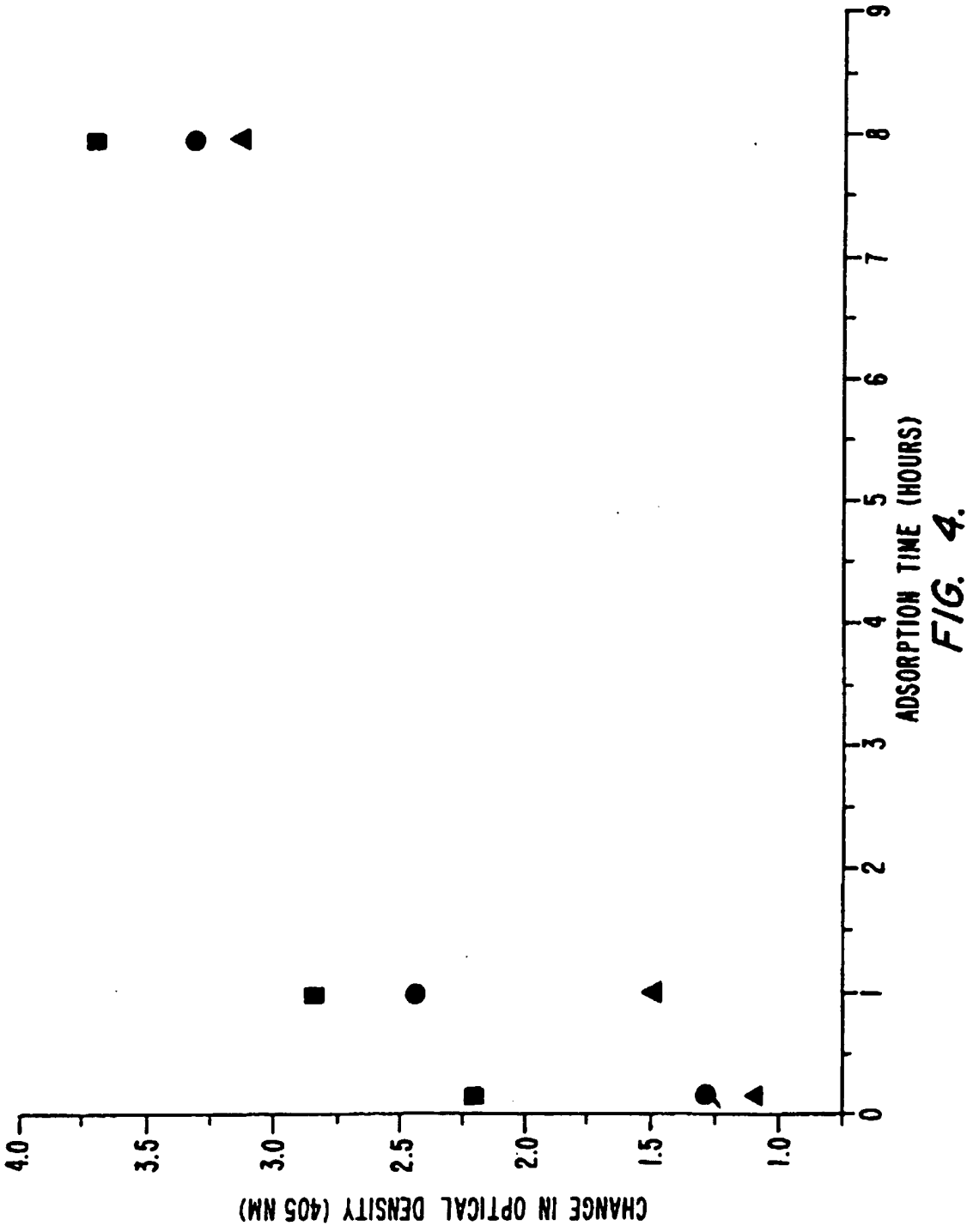
DESORPTION TIME (HOURS)
0.16
1.0
8.0
CHANGE IN OPTICAL DENSITY (405 NM)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0
1
2
3
4
5
6
7
8
9
ADSORPTION TIME (HOURS)

FIG. 4.

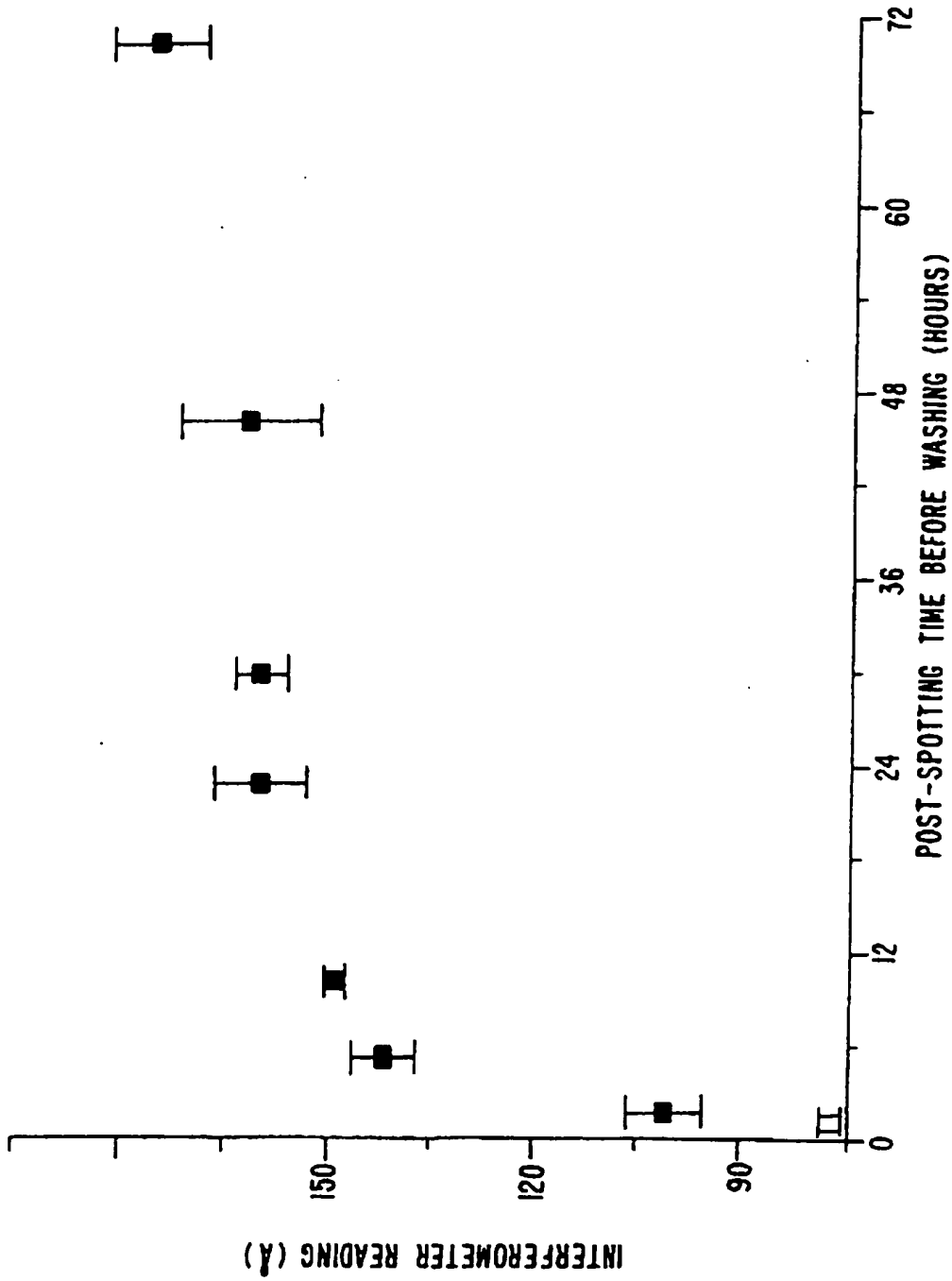
INTERFEROMETER READING (Å)
150
120
90
0
12
24
36
48
60
72
POST-SPOTTING TIME BEFORE WASHING (HOURS)

FIG. 5.

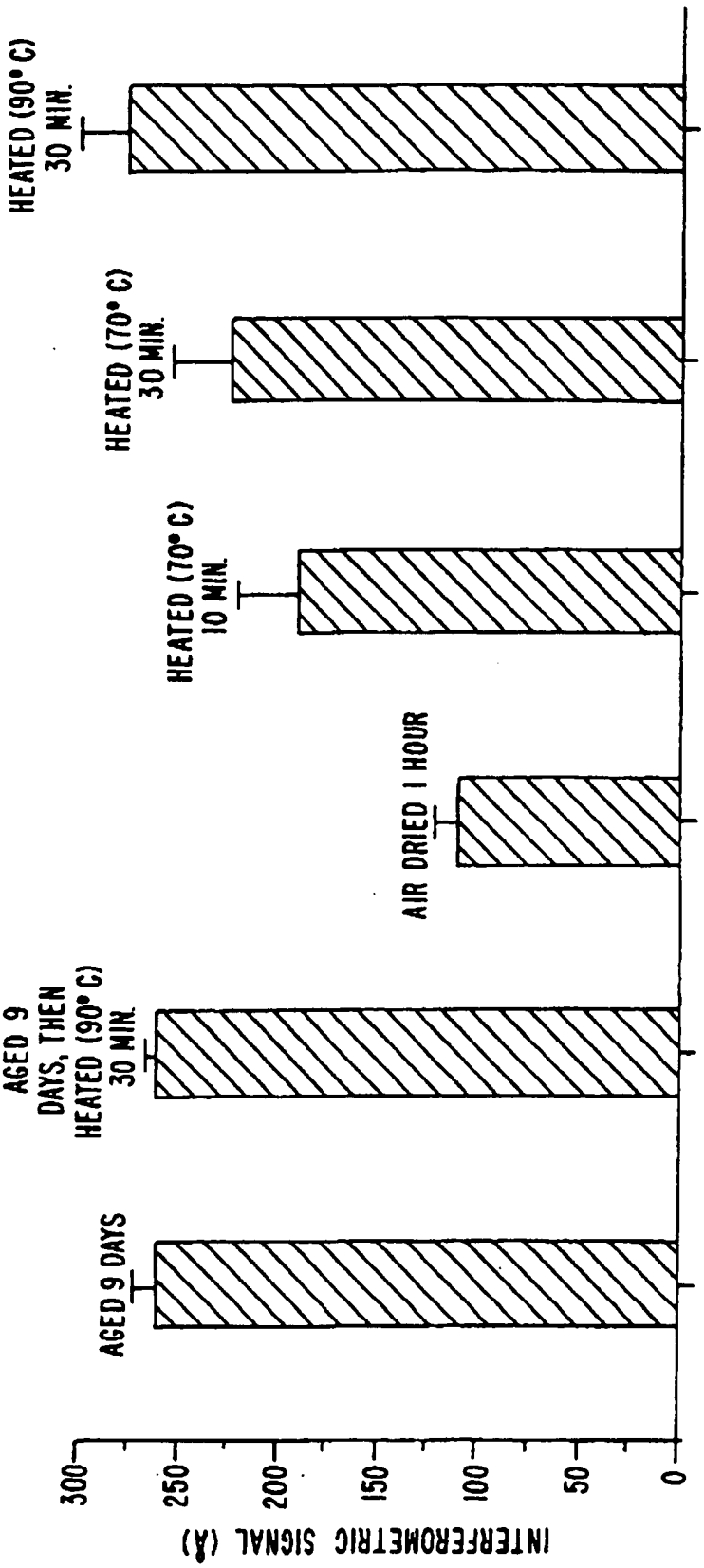
INTERFEROMETRIC SIGNAL (Å)
300
250
200
150
100
50
0
AGED 9 DAYS
AGED 9 DAYS, THEN HEATED (90° C) 30 MIN.
AIR DRIED 1 HOUR
HEATED (70° C) 10 MIN.
HEATED (70° C) 30 MIN.
HEATED (90° C) 30 MIN.

FIG. 6.

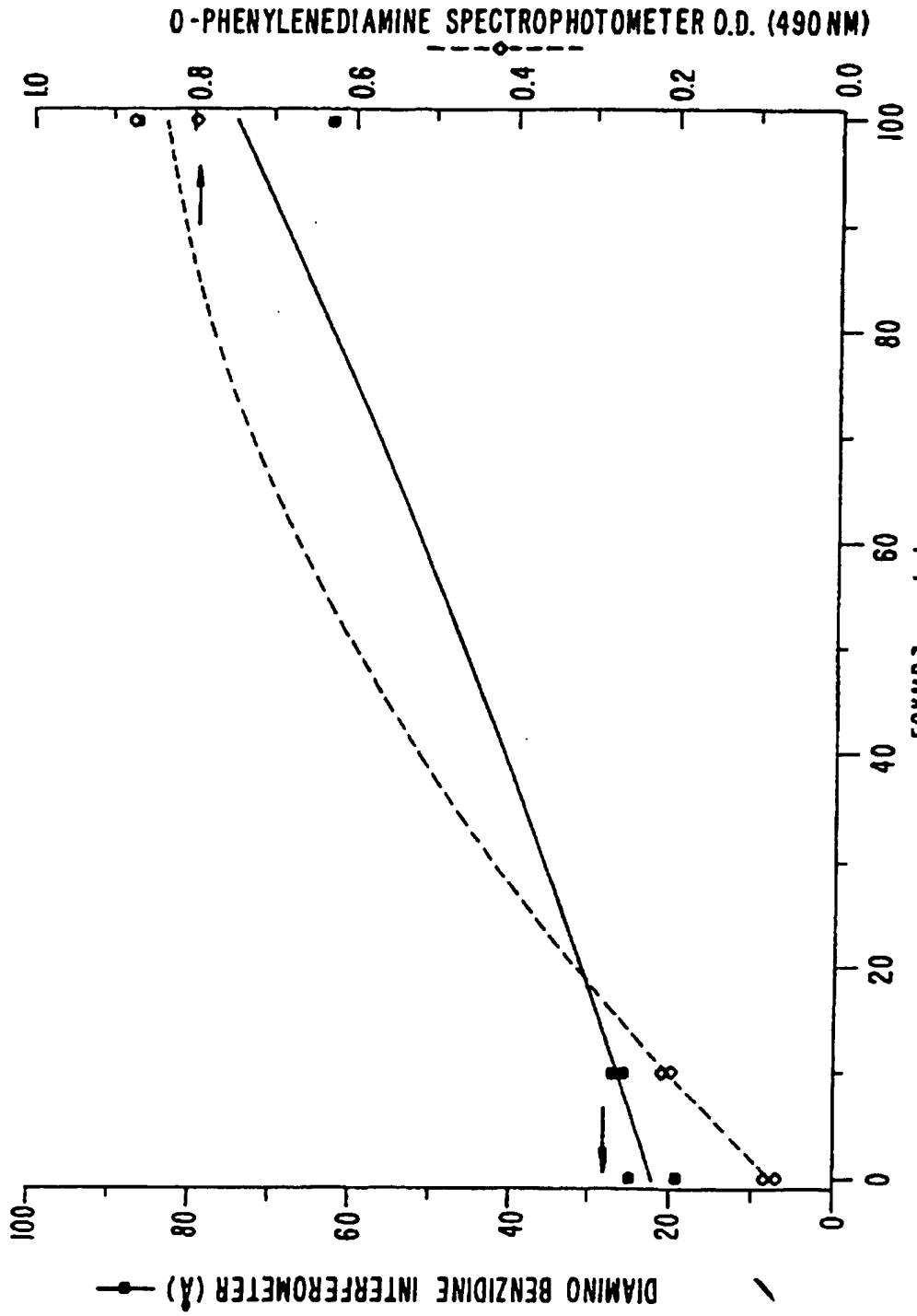
O-PHENYLENEDIAMINE SPECTROPHOTOMETER O.D. (490 NM)
1.0
0.8
0.6
0.4
0.2
0.0
100
80
60
40
20
0
[CKMB] ng/mL
DIAMINO BENZIDINE INTERFEROMETER (A)

FIG. 7.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00416

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/543
US CL :436/518, 524, 527, 528, 530, 532, 543; 422/57; 435/7.1, 805, 970
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/518, 524, 527, 528, 530, 532, 543; 422/57; 435/7.1, 805, 970

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,362,655 A (SCHENK ET AL) 08 NOVEMBER 1994, column 1, lines 52-61.	1-41
Y	US 4,791,067 A (SHEIMAN ET AL) 13 DECEMBER 1988, column 3, line 6- column 4, line 28.	1-41
X --- Y	US 5,045,480 A (JOHNSON ET AL) 03 SEPTEMBER 1991, columns 9-12.	1-6, 12-14, 31-37, 39-41 --- 7-11, 15-30, 38
Y	US 5,316,784 A (MAURER ET AL) 31 MAY 1994, columns 2-3.	1-41
Y	US 5,063,081 A (COZZETTE ET AL) 05 NOVEMBER 1991, columns 31-32, and columns 50-51.	1-41

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
24 APRIL 1996	10 MAY 1996

Name and mailing address of the ISA/US	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00416

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,960,692 A (LENTRICHIA ET AL) 02 OCTOBER 1990, column 3, lines 23-38.	1-41
A	US 5,279,955 A (PEGG ET AL) 18 JANUARY 1994, column 3, lines 19-29.	1-41
X,P --- Y,P	US 5,468,649 A (SHAH ET AL) 21 NOVEMBER 1995, see abstract.	1-2, 4, 5, 12, 31-33, 35-36 --- 8-11, 15-23, 25-26, 28
X --- Y	US 4,096,138 A (SCHERR) 20 JUNE 1978, column 4, lines 21-37, and column 9, lines 50-68 .	1-5, 13-14, 31, 33-36, 40 --- 7-12, 15-26, 28-30, 32, 37, 39, 41

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*